Temperature-Modulated Immunogenicity to *Yersinia pestis* from *Yersinia enterocolitica* O3

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The ability of *Yersinia enterocolitica* O3, grown at 25°C, to promote cross-immunity to *Y. pestis* was lost after repeated subcultures at 37°C, which selected for bacterial populations having lower in vivo survival. Subculturing *Y. enterocolitica* O3 from 37 to 25°C restored the cross-immunogenicity although the in vivo survival remained low.

Mice convalescing from *Yersinia enterocolitica* O3 are resistant to *Y. pestis* (1, 3). It has been reported that incubation of *Y. enterocolitica* O3 grown at 37°C rather than 25°C decreases its in vivo survival in mice (12) and could inhibit the elaboration of common protein antigens to *Y. pestis* (4). With respect to these temperature-dependent parameters, we tested, in this study, whether the ability of *Y. enterocolitica* O3 to promote immunity to *Y. pestis* was influenced by the temperature of culture of the *Y. enterocolitica* O3 inoculum or not.

Groups of Swiss female mice, (Institut Pasteur, Ferme Expérimentale de Rennemoulin, Villepreux, France), 5 to 6 weeks old, were injected intravenously with *Y. enterocolitica* 4052 (YE) as described in previous studies (1, 2) (mean 50% lethal dose, 7 × 10⁷ colony-forming units [CFU]); YE was grown for 24 h at 25 or 37°C on Trypto-casein-soy agar (Institut Pasteur Production). Mice that were convalescing from YE infection were challenged with *Y. pestis* 6/69M (YP) as described previously (1, 3) (mean 50% lethal dose, <10 CFU); YP grown on Trypto-casein-soy agar at 25°C was injected subcutaneously.

In this first experiment in this study, mice injected with YE grown at 25 or 37°C (six repeated subcultures at 37°C) were checked for their immunity to YP by testing their acquired resistance and by measuring their delayed-type hypersensitivity to 5 × 10⁶ heat-killed YP injected subcutaneously in a hind footpad, as previously described (9). Mice convalescing from YE infection, induced with the inoculum grown at 25°C, exhibited acquired resistance and delayed-type hypersensitivity to YP, whereas mice convalescing from YE infection, induced with a threefold-higher inoculum grown at 37°C, were susceptible and did not express delayed-type hypersensitivity to YP (Table 1).

It may be argued that the inability of YE subcultured at 37°C to induce cross-immunity to YP might be because of a lower and short-time in vivo survival of the inoculum (12). In the next experiment, mice were injected intravenously with decreasing doses of YE grown at 25°C or with approximately the same dose of YE subcultured four or six times at 37°C; the in vivo survival of each inoculum was measured by counting the number of CFU recovered per spleen, homogenized, sampled on Trypto-casein-soy agar, and incubated at 28°C for 48 h, as previously described (2). By decreasing doses of YE grown at 25°C, we induced splenic infection, the intensity of which was in correlation with the size of each inoculum (Fig. 1). The injection of YE repeatedly subcultured at 37°C induced splenic infection, the intensity of which decreased depending on the number of subcultures at 37°C (Fig. 1). A low dose of YE grown at 25°C (3 × 10⁶ CFU) or a heavy dose of YE subcultured six times at 37°C (1.6 × 10⁹ CFU) induced quantitatively equivalent splenic infection; however, after mice convalescing from either the low or heavy dose of YE were challenged with YE (30 times the 50% lethal dose, at day 30 after the YE injection), survivors were observed only in the group of 10 mice that were first infected with 3 × 10⁹ CFU of YE grown at 25°C (four survivors at day 20 after YP challenge), whereas the 10 mice that were first infected with 1.6 × 10⁹ CFU of YE subcultured six times at 37°C and the 10 uninfected control mice died within 10 days.

Thus, it seemed that repeated subcultures at 37°C not only decreased the in vivo survival rate of YE, but moreover might have removed cross-protection to YP.

Among the *Y. pestis* virulence determinants (5), VW antigens have been identified in some virulent *Y. enterocolitica* (6, 7). Since the pres-
TABLE 1. Effect of culture temperature on acquired resistance and delayed-type hypersensitivity to YE of mice convalescing from YE

<table>
<thead>
<tr>
<th>YE inoculum</th>
<th>Acquired resistance to YE lethal challengea</th>
<th>Delayed-type hypersensitivity to YE footpad swelling (mm)b at:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Survivors/totalc</td>
<td>Mean survivald</td>
</tr>
<tr>
<td>5 × 10⁵ CFU of YE grown at 25°C</td>
<td>8/10</td>
<td>0.74 ± 0.09f</td>
</tr>
<tr>
<td>1.7 × 10⁶ CFU of YE subcultured</td>
<td>0/10</td>
<td>0.51 ± 0.03f</td>
</tr>
<tr>
<td>6 times at 37°C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uninfected control mice</td>
<td>0/10</td>
<td>0.47 ± 0.08f</td>
</tr>
</tbody>
</table>

a 30 times the 50% lethal dose injected subcutaneously at day 30 after YE injection.
b Mean value ± standard error of the footpad swelling measured at 24 h after the injection of 5 × 10⁶ CFU of heat-killed YE in groups of 5 mice.
c Number of surviving mice at day 20 after YE challenge per total number of mice tested.
d Mean for 10 mice ± standard error of the mean, calculated after negative-exponential transformation of Liddel (11), using the constant θ = 0.1 and the full period of observation at 20 days (T = 20).
e Significantly higher than controls at P < 10⁻⁴, determined by Student’s t test.

ence of VW antigens is correlated with the inability of *Yersinia* spp. to grow on magnesium oxalate agar at 37°C, we tested the growth of various YE cultures by the method of Higuchi and Smith (8) compared with that of the *Y. enterocolitica* WA strain, kindly provided by P. B. Carter (The Trudeau Institute, Saranac Lake, NY); the *Y. enterocolitica* WA strain was stored at 25°C and used as a reference VW positive strain (6). YE colonies were counted at equivalent frequencies on both magnesium oxalate and Trypto-casein-soy agar plates incubated at 25 or 37°C, after 1.5 × 10⁵ to 2.5 × 10⁶ CFU of YE grown at 25°C or subcultured at 37°C had been sampled.

Laird and Cavanaugh reported that virulent *Yersinia* spp. autoagglutinated in tissue culture media incubated at 36°C (10); they studied *Y. enterocolitica* 4052, the same strain we used in the present experiments, and found positive virulent and negative avirulent colonies. Accordingly, isogenic pairs from 10 randomly selected colonies of YE grown at 25°C or subcultured from 25 to 37°C, then from each repeated subculture at 37°C until the 20th subculture, and finally from YE subcultured 20 times at 37°C then once at 25°C were tested for their ability to autoagglutinate when grown in RPMI-1640 medium containing 25 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffer (Flow Laboratories, 92600 Assinières). No positive virulent colonies were identified after the fourth subculture at 37°C or after the subcultures from 37 to 25°C.

Thus, it seemed that subcultures at 37°C selected for non-autoagglutinating avirulent YE (Fig. 1) were unable to promote cross-protection or delayed-type hypersensitivity to YE (Table 1). We tested whether these temperature dependence-associated properties were stable by comparing the in vivo survival rate and the ability to induce resistance to YE of YE grown at 25°C, YE subcultured 20 times at 37°C, and YE subcultured 20 times at 37°C then once at 25°C. When YE was subcultured from 37 to 25°C, the in vivo survival rate remained as low as that for YE subcultured 20 times at 37°C, but a significant, although partial, cross-protection to YE was restored (Table 2).

Culture of *Y. enterocolitica*, including O3 strains, at 37°C in artificial media without the addition of calcium may lead to the lack of plasmid-associated virulence correlated with autoagglutination (13, 14). From our results, it appeared that such a phenomenon may have occurred and could explain the lower in vivo survival of YE inocula subcultured at 37°C. The

![Graph showing kinetics of Y. enterocolitica per spleen](http://iai.asm.org/)

**FIG. 1.** Kinetics of the in vivo survival in mice injected intravenously with cultures derived from YE grown at 25°C: 1.8 × 10⁶ (●), 5 × 10⁶ (■), 3 × 10⁶ (□), or 1.4 × 10⁸ CFU of YE subcultured four times at 37°C (●) or six times at 37°C (□). Values are expressed as the means ± standard errors for five mice.
### TABLE 2. Comparison between the autoagglutination property, the in vivo survival, and the ability to promote acquired resistance to YP of various cultures derived from YE

<table>
<thead>
<tr>
<th>YE culture</th>
<th>Autoagglutination</th>
<th>Intravenous inoculum (log₁₀ CFU)</th>
<th>Log₁₀ CFU recovered per spleen at:</th>
<th>Acquired resistance to YP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Day 1</td>
<td>Day 2</td>
<td>Day 3</td>
</tr>
<tr>
<td>YE25</td>
<td>9/20</td>
<td>5.78</td>
<td>4.80 ± 0.08</td>
<td>4.83 ± 0.06</td>
</tr>
<tr>
<td>YE37</td>
<td>0/20</td>
<td>5.80</td>
<td>3.11 ± 0.10</td>
<td>2.93 ± 0.14</td>
</tr>
<tr>
<td>YE37–25</td>
<td>0/20</td>
<td>5.60</td>
<td>3.01 ± 0.09</td>
<td>2.86 ± 0.08</td>
</tr>
<tr>
<td>Uninfected control mice</td>
<td>0/20</td>
<td>3.11 ± 0.09</td>
<td>2.86 ± 0.08</td>
<td>2.33 ± 0.17</td>
</tr>
</tbody>
</table>

- YE25, YE grown at 25°C; YE37, YE subcultured 20 times at 37°C; and YE37–25, YE subcultured 20 times at 37°C then once at 25°C.
- Number of positive colonies per total number of colonies.
- Mean ± standard error of five mice.
- Number of surviving mice at day 20 after YE injection.
- Mean ± standard error for 19 mice, calculated after negative-exponential transformation of Liddel (11), using $\theta = 0.1$ and $T = 20$.
- *Significantly higher than YE37 at $P < 10^{-4}$, determined by Student's t test.

ability to promote cross-immunity to YE, which was recovered after one subculture from YE subcultured from 37 to 25°C, might be due to a phenotypical property, perhaps an enhanced production by YE grown at a low temperature, of unidentified common antigen to YE.

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### LITERATURE CITED